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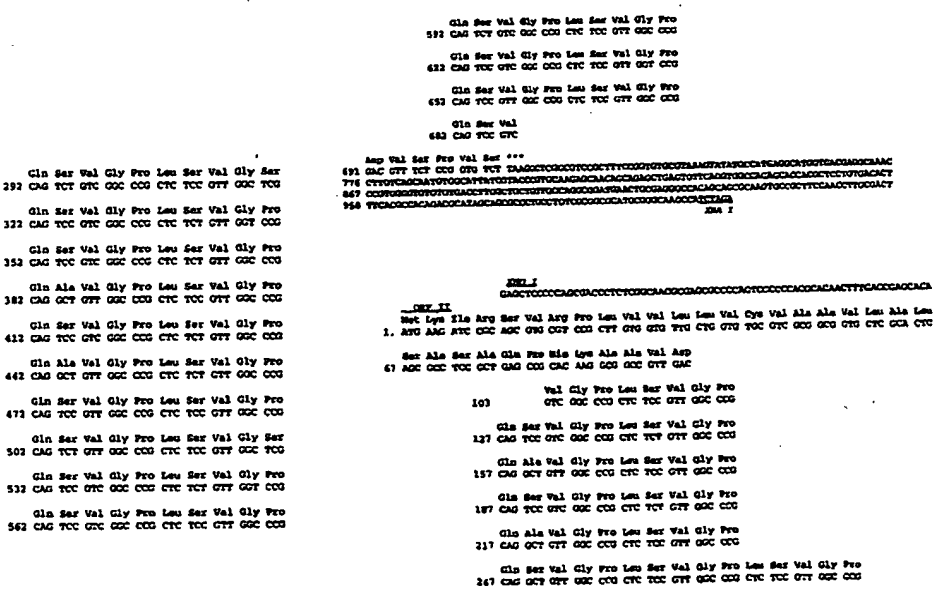
(54) Title: DIFFERENTIALLY EXPRESSED *LEISHMANIA* GENES AND PROTEINS



(57) Abstract

Differentially expressed *Leishmania* genes and proteins are described. One differentially expressed gene (A2) is expressed at significantly elevated levels (more than about 10 fold higher) in the amastigote stage of the life cycle when the *Leishmania* organism is present in macrophages than in the free promastigote stage. The A2 gene encodes a 22 kD protein (A2 protein) that is recognized by kala-azar convalescent serum and has amino acid sequence homology with an S-antigen of *Plasmodium falciparum* Vietnamese isolate VI. Differentially expressed *Leishmania* genes and proteins have utility as vaccines, diagnostic reagents, as tools for the generation of immunological reagents and the generation of attenuated variants of *Leishmania*.

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TITLE OF INVENTION

DIFFERENTIALLY EXPRESSED LEISHMANIA GENES AND PROTEINS

FIELD OF INVENTION

The present invention is related to molecular cloning of Leishmania genes and, in particular, to the cloning of amastigote differentially expressed genes from Leishmania donovani.

BACKGROUND TO THE INVENTION

Leishmania potozoans are the causative agents of human leishmaniasis, which includes a spectrum of diseases ranging from self-healing skin ulcers to fatal visceral infections. Human leishmaniasis is caused by at least thirteen different species and subspecies of parasites of the genus Leishmania. Leishmaniasis has been reported from about eighty countries and probably some 400,000 new cases occur each year. Recently, the World Health Organization has reported 12 million people to be infected (ref. 1 - a listing of the references appears at the end of the disclosure).

L. donovani causes visceral leishmaniasis, also known as kala-azar. L. brasiliensis causes mucotaneous leishmaniasis and L. major causes cutaneous leishmaniasis. Untreated visceral leishmaniasis is usually fatal and mucocutaneous leishmaniasis produces mutilation by destruction of the naso-oropharyngeal cavity and, in some cases, death.

In addition, a major health problem has been created in areas of high infection when blood is collected for transfusion purposes. Since blood is a carrier of the parasites, blood from an infected individual may be unknowingly transferred to a healthy individual.

The Leishmania protozoans exist as extracellular flagellated promastigotes in the alimentary tract of the sandfly in the free-living state, and are transmitted to the mammalian host through the bite of the insect vector. Once introduced, the promastigotes are taken up by

macrophages, rapidly differentiate into non-flagellated amastigotes and start to multiply within the phagolysosomal compartment. As the infected cells rupture, amastigotes subsequently infect other macrophages giving rise to the various symptoms associated with leishmaniasis (refs. 1 and 2). In this manner, it is the amastigote form of the parasite which is responsible for the pathology in humans.

While in the midgut of the insect, newly transformed promastigotes, functionally avirulent, progressively acquire capacity for infection and migrate to the mouthparts (ref. 3). This process, termed the metacyclogenesis, which occurs only in promastigotes, is concurrent with the differential expression of major surface glycoconjugates which mediate the migration of promastigotes in the alimentary tract and prepare the organism for the serum environment (refs. 4 and 5). In comparison, the promastigote to amastigote cytodifferentiation is a profound morphological and physiological transformation. During the promastigote to amastigote differentiation, the parasite looses its flagellum, rounds-up, changes its glycoconjugate coat (refs. 6, 7 and 8) and expresses a set of metabolic enzymes optimally active at low pH. The survival of the parasite inside the macrophage phagolysosome is associated with its ability to down-regulate many effector and accessory functions of its host cell, including oxygen metabolite-mediated killing and the capacity of the macrophage to act as an efficient antigen presenting cell (reviewed in, for example, ref. 9).

Leishmaniasis is, therefore, a serious disease and various types of vaccines against the disease have been developed, including live parasites; frozen promastigotes from culture; sonicated promastigotes; gamma-irradiated live promastigotes; and formalin-killed promastigotes treated with glucan (reviewed in, for example, ref. 10).

However, none of these approaches have provided satisfactory results.

The promastigote-amastigote differentiation is important to the establishment of infection. It would be desirable to identify genes and gene products that are differentially expressed when the amastigotes are present in macrophages.

Joshi, et al. describe L. donovani genes that are expressed at about two-fold higher in in vitro generated and maintained "amastigotes" compared to promastigotes (ref. 11).

SUMMARY OF THE INVENTION

The present invention is directed towards the provision of a Leishmania protein that is differentially expressed in the amastigote stage when the Leishmania organism is present within macrophages and genes encoding the differentially expressed protein. The amastigote differentially expressed gene and protein are useful for the preparation of vaccines against disease caused by Leishmania, the diagnosis of infection by Leishmania and as tools for the generation of immunological reagents and the generation of attenuated variants of Leishmania.

In accordance with one aspect of the present invention, there is provided a purified and isolated DNA molecule, the molecule comprising at least a portion coding for a differentially expressed gene of a Leishmania organism, the differentially expressed gene being expressed at an increased level when the amastigote form of the Leishmania organism is present within a macrophage. The increased level of expression maybe at least about a ten-fold increase in expression. In one embodiment of the present invention, the differentially expressed gene may be a virulence gene of the Leishmania organism and may be required for maintenance of infection by the amastigote form of the Leishmania organism.

In a further aspect of the invention, the differentially expressed virulence gene is functionally disabled by, for example, deletion or mutagenesis, such as insertional mutagenesis, to produce an attenuated Leishmania organism for use as, for example, a live vaccine. Conveniently, strains of Leishmania from which differentially expressed genes may be isolated include Leishmania donovani.

Further aspects of the invention include the protein encoded by the differentially expressed gene, and the use of the protein in vaccination and diagnosis. Additional aspects of the invention include an attenuated strain of Leishmania in which the virulence gene is disabled and a vaccine comprising the same.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic outline of the amastigote cDNA library construction and differential screening with amastigote and promastigote-specific cDNA probes. An example of an amastigote-specific cDNA clone is indicated by an arrow on the colony hybridization autoradiogram;

Figure 2 shows a restriction enzyme and size analysis of Leishmania donovani amastigote-specific cDNA clones;

Figure 3 shows a Southern blot analysis of Leishmania donovani amastigote-specific cDNA clones;

Figure 4 shows a Northern blot analysis to demonstrate that A2-specific transcripts are present in amastigote-infected macrophages but not promastigotes;

Figure 5 shows a Southern blot analysis to demonstrate that A2 transcripts are encoded by a multigene family;

Figure 6 shows a restriction map of plasmid pGECO 90 that contains the L. donovani A2 gene;

Figure 7 shows a restriction map of a genomic clone of the A2 gene and its relationship to A2-related cDNAs;

Figure 8 shows the nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the open reading frame II (ORF II) of the Leishmania donovani A2 gene;

Figure 9 shows the homology between the Leishmania donovani A2 protein (SEQ ID NO: 2) and the Plasmodium falciparum S antigen (SEQ ID NO: 3) within the repeated subunits of these proteins;

Figure 10 shows the construction of a plasmid pET 16b/ORF II$^+$ for expression of the A2 protein;

Figure 11 shows the presence of antibodies against A2 fusion protein in kala-azar immune serum by immunoprecipitation;

Figure 12 shows the specific recognition of A2 fusion protein by kala-azar sera by Western blot analysis; and

Figure 13 shows the results of Southern blot analysis of the separated chromosomes of different species and subspecies of Leishmania.

GENERAL DESCRIPTION OF THE INVENTION

Referring to Figure 1, there is illustrated a method used for isolating a Leismania gene differentially expressed during the amastigote stage in the life cycle thereof. The method comprises the steps of (a) constructing a cDNA library from the Leishmania organism in the amastigote stage in the life cycle thereof; (b) constructing a first mixture of cDNA probes specific for the amastigote stage in the life cycle; (c) constructing a second mixture of cDNA probes specific for the promastigote stage in the life cycle; (d) separately probing the cDNA library with the amastigote and promastigote-specific cDNA probes in order to identify cDNA clones that are recognized by the amastigote mixture of cDNA probes but not the promastigote mixture of cDNA probes; and (e) isolating the cDNA clones identified in step (d).

The amastigote-specific cDNA clones identified by the above procedure can be further characterized by restriction enzyme analysis and their relatedness determined by Southern hybridization studies. To determine if cDNA clones identified by the above procedure represent amastigote-specific clones that are expressed at a higher level (more than about ten-fold higher) when the amastigote form of the Leishmania organism is present within macrophages, macrophages were infected with amastigotes and differentially-expressed gene transcripts were detected by Northern blot analysis. In an embodiment of the present invention, the differentially expressed Leishmania gene is L. donovani gene that is expressed at an increased level when the amastigote form of the Leishmania organism is present within a macrophage. The intracellular environment of the macrophage has an acidic pH of, for example, about 4.5. The differentially expressed genes include those having sequences, such as the DNA sequence set out in Figure 8 (SEQ ID No: 1) or its complementary strand; and DNA sequences which hybridize under stringent conditions to such DNA sequences. Such differentially expressed gene sequences include the A2 gene of L. donovani having the DNA sequence set out in Figure 8 and the invention includes a cDNA clone encoding the A2 gene depicted in Figure 8, which clone may be in the form of a plasmid, particularly that designated pGEC0 90 (Figure 6), which has ATCC accession number ATCC 75510.

The differentially expressed genes may encode proteins, such as the 22 kD A2 protein (SEQ ID No: 2), being encoded by the longest open reading frame (ORF II) of the A2 gene. Most of the predicted A2 protein is composed of a repetitive sequence consisting of a stretch of ten amino acids repeated nineteen times (Figure 8). Since each unit of this repeat contains two serines, two valines, two leucines and two prolines separated from

each other by five residues, the repeated region also may be considered as a stretch of five amino acids repeated thirty-eight times. The amino acid sequence of the A2 protein has homology with an S-antigen of Plasmodium falciparum (SEQ ID NO: 3), as shown in Figure 9. As with the L. donovani A2 protein, the carboxy-terminal portion of the S-antigen of P. falciparum Vietnamese isolate VI is composed of a stretch of eleven amino acids repeated nineteen times; the repeated units of both proteins are 50% identical and 80% homologous.

Life cycle stage specific genes from Leishmania may be isolated in the present invention. Some of these genes are required for transition between the life cycle stages and include virulence genes of the Leishmania parasite, such as virulence genes that are required for maintenance of infection by the amastigote form of the Leishmania organism. These virulence genes may be functionally disabled by, for example, deletion or mutation, including insertional mutagenesis and, furthermore, the wild-type Leishmania gene may be replaced by the functionally disabled gene. The virulence genes may be functionally disabled by, for example, replacing the A2 gene by a selectable antibiotic resistance gene by homologous recombination following transformation of the Leishmania organism with a fragment of DNA containing the antibiotic resistance gene flanked by 5'- and 3'- non-coding DNA sequences. This process can be used to generate attenuated variants of Leishmania and the residual pathogenicity of the attenuated variants can be assessed in mice and hamsters pigs. It is likely that deletion of genes that are selectively expressed in the human host environment (that being when the Leishmania organism is inside the macrophage cell) result in an attenuated strain which cannot survive in humans but generates a protective immune response. Attenuated strains of Leishmania would be useful as live vaccines

against the diseases caused by Leishmania and such attenuated strains form an aspect of the present invention.

Differentially expressed genes and proteins of Leishmania typified by the embodiments described herein are advantageous as:

- antigens for vaccination against the diseases caused by Leishmania.
- diagnostic reagents including hybridization probes, antigens and the means for producing specific antisera for (for example) detecting infection by Leishmania.
- target genes for functional disablement for the generation of attenuated Leishmania variants.

Vaccines comprising an effective amount of the protein encoded by the differentially expressed genes or of an attenuated strain of Leishmania and a physiologically-acceptable carrier therefor may utilize an adjuvant as the carrier and the protein may be presented to the immune system of the host in combination with an ISCOM or liposome. The vaccine may be formulated to be administered to a host in an injectable form, intranasally or orally, to immunize the host against disease.

BIOLOGICAL DEPOSITS

A plasmid pGECO 90 described and referred to herein was deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland, USA, pursuant to the Budapest Treaty on July 28, 1993 and prior to the filing of this application and assigned the ATCC accession number 75510. A diagram of this plasmid is shown in Figure 6. The plasmid contains the A2 gene of L. donovani described herein. The plasmid will become available to the public upon grant of a patent based upon this United States patent application. The invention

described and claimed herein is not to be limited in scope by the material deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent materials are within the scope of the invention.

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics and protein biochemistry used but not explicitly described in this disclosure and these Examples, are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example describes culturing and isolation of Leishmania organisms.

Amastigotes of the L. donovani Ethiopian LV9 strain were harvested from spleens of infected female gold Symian hamsters and purified as described previously (ref. 12). Briefly, parasites were released from tissue using an homogenizer, the mixture was centrifuged three times at 100xg to remove cellular debris, and amastigotes were pelleted at 1500xg. The pellet was resuspended in 0.17 M sodium acetate to lyse contaminating red blood cells and amastigotes were recovered by centrifugation at 1500xg. Organisms were incubated at 37°C in complete RPMI medium (RPMI 1640 supplemented with 10% endotoxin free heat-inactivated FBS, 10 ml of 1M HEPES pH 7.3, 100

U of penicillin and 100 U of streptomycin per ml) for 18 hours prior to RNA extraction. After this period of incubation and multiple washes, the amastigote preparation was still physiologically active and relatively free of host cell contamination. To obtain promastigotes, LV9 strain amastigotes were allowed to differentiate in complete RPMI medium at 26°C, and cultured for at least seven days in the same medium before use (ref. 12).

Promastigotes of the L. donovani Sudanese strain 1S2D were cultivated and passaged in complete RPMI medium at 26°C. Amastigote-like organisms of the 1S2D strain were cultivated as described by Doyle et al. (ref. 13). The Sudanese strains 1S2D and 1S2D (wt) were obtained from Dr. S. Turco, the University of Kentucky, USA. The 1S2D (wt) promastigotes were adapted to grow in axenic conditions and had lost the ability to transform into infective promastigotes.

Example 2

This Example describes the preparation of and screening of a Leishmania cDNA library.

A method for isolating a Leishmania gene differentially expressed during the amastigote stage in the life cycle of the organism is illustrated in Figure 1.

Total RNA of amastigotes and promastigotes was prepared by the guanidinium isothiocyanate method using "RNAzol" (Trademark of Cinna/biotecx Laboratories International Inc., Friendswood, TX); poly A^+ RNA was selected by oligo dT cellulose chromatography (grade 7:Pharmacia) as described by Sambrook et al. (ref. 14). A total of 10 μg of amastigote mRNA was used to construct an Eco RI/ Xho I unidirectional cDNA library of 10^6 clones in the "λ ZAP II" vector (Trademark of Stratagene); hemi-methylated cDNA was produced using the manufacturers reagents and protocols. About 40,000

amastigote and promastigote-specific clones of the primary library were screened differentially with amastigote and promastigote stage-specific gene probes. The cDNA probes were prepared using oligo dT_{12-18} primer (Pharmacia) and M-MLV reverse transciptase (BRL) following protocols previously described (ref. 15). Duplicate filters were hybridized with each probe for 18 h at 42°C in 50% formamide, 6X SSC, 5X Denhardt's solution, 5% dextran sulfate. Membranes then were washed twice at room temperature in 1X SSC for 20 min, twice at 55°C in 1X SSC, 0.1% SDS and then autoradiographed on "X-OMAT" films (Trademark of Kodak) with an intensifying screen for 18 to 72 hours. Such washing operation corresponds to stringent conditions of hybridization. Areas on the plates containing putative clones of interest were picked and the phage pools were submitted to a second round of screening. An example of an amastigote-specific cDNA clone is indicated by the arrow on the plaque hybridization autoradiogram of Figure 1.

Although cDNA clones representing promastigote-specific transcripts were more abundant than clones representing amastigote-specific transcripts, seven independent cDNA clones which only hybridized with amastigote-specific probes were isolated and termed 2, 3, 5, 6, 8, 9, 11. For each cDNA clone isolated, a Bluescript plasmid derivative was excised from the λZAP II recombinant phages in vivo using the helper phage R-408.

Example 3

This Example describes the characterization of amastigote-specific cDNA clones.

The insert size of each of the Bluescript plasmids corresponding to the amastigote-specific cDNA clones was determined by restriction enzyme digestion and agarose gel electrophoresis (Figure 2). Recombinant plasmids (A2, A3, A5, A6, A8, A9 and A11) were digested with Eco

RI and Xho I to excise the cDNA inserts. Fragments were separated on a 1% agarose gel and stained with ethidium bromide. The cDNA inserts varied from 0.5 kb (A5) to 1.8 kb and A8 contained an internal Eco RI site. To determine if the amastigote-specific cDNA clones contain common sequences, Southern blot hybridization analysis of the Bluescript plasmids corresponding to the amastigote-specific cDNA clones was performed using clone A2 and clone A6 specific probes (Figure 3).

For Southern blot analysis, 10 μg of total DNA was cut to completion with the restriction enzymes Eco RI and Xho I and separated on a 1% agarose gel. The restriction fragments were transferred to nylon membranes using standard procedures (ref. 16) and duplicates hybridized with α-^{32}P dCTP nick-translated probes representing the inserts of the cDNA clones A2 (0.9kb) or A6 (0.6kb). The A2 probe recognized five cDNAs (A2, A3, A8, A9 and A11) and the A6 cDNA only hybridized to itself. Thus, this Southern blot analysis indicated that cDNA clones A2, A3, A8, A9 and A11 contained homologous sequences but A5 and A6 were clones of unrelated amastigote-specific transcripts.

To confirm that the A2 series of clones represented Leishmania genes that were differentially expressed when the Leishmania organism is present in macrophages compared to expression in the free-living promastigotes, Northern blot analysis was performed. Total RNA was extracted from bone marrow-derived macrophages (BMM), L. donovani LV9-infected BMM (IBMM) and L. donovani LV9 promastigotes (PRO). Murine bone marrow-derived macrophage cultures and L. donovani amastigote in vitro infections were carried out as previously described (ref. 12). The RNA species (15 μg) were separated on an agarose gel and stained with ethidium bromide prior to transfer (Figure 4, right panel). The RNA was denatured by glyoxal treatment and transferred to a nylon membrane.

The Northern blot was hybridized with labelled cDNA A2 (0.9 kb) fragment, as previously described (ref. 12) (Figure 4, left panel). This probe recognized predominantly a 3.5 kb transcript present in amastigote-infected macrophages but not in promastigotes or in non-infected macrophages. This analysis showed that the A2 gene was differentially expressed at an increased level in amastigotes when they were present in macrophages compared to a free-living existence and that the increased expression was at least a ten fold increase.

Example 4

This Example describes the genomic arrangement and sequencing of the Leishmania donovani amastigote-specific A2 gene.

Regulation of transcription is one of the unusual features of the genetics of trypanosomatids. Copies of a gene or related genes are often clustered in tandem arrays on the same chromosome and a unique promoter region regulates expression of the cluster. Transcription leads to the synthesis of a polycistronic RNA molecule which is cleaved into monomeric units by trans-splicing prior to translation. The genomic arrangement of A2 related gene(s) was investigated by Southern blot analysis to determine whether it represents a multigene family. Total DNA was digested to completion with several restriction enzymes (E: Eco RI, S: SalI, X: Xba I, C: Cla I, P: Pvu II). For double digests, the DNA was first cut to completion with Cla I or Pvu II, the DNA precipitated and resuspended in the appropriate buffer for the second digestion. Restriction fragments were separated on a 0.7% agarose gel, transferred to a nylon membrane and hybridized with a 0.5 kb Pst I/Xho I fragment of the A2 cDNA insert nick-translated with α-^{32}P dCTP. For each digest, the hybridization pattern displayed a series of bands of different intensities, clearly showing that many copies of the gene were present

in the genome (Figure 5). Moreover, common bands at about 6 to 8 kb for the Eco RI, Xba I and Sal I digests suggested an arrangement in tandem arrays. However, the presence of at least two other bands in each lane suggested that more than one cluster existed, each cluster being flanked by restriction fragments of different sizes. Alternatively, clusters also may carry copies of unrelated genes or intergenic regions of variable sizes.

To identify the protein coding region of A2, genomic clones carrying the A2 gene sequence were isolated. A partial genomic library containing 6 to 10 kb Eco RI fragments was constructed in the lambda ZAP II vector (Stratagene). More than 2,000 clones were screened on duplicate filters with probes prepared with the A2 cDNA using techniques and hybridization conditions described in Example 2. Eight clones were isolated and purified. Bluescript plasmid derivatives were excised from recombinant λ phages as for cDNA clones.

The 1.9 kb Xho I/ Eco RI insert fragment of the A2 Bluescript clone was subcloned into the Bluescript phagemids KS^+ and KS^- for sequencing. Nested deletions were carried out on both plasmids using Exo III exonuclease and S1 nuclease. Sequencing reactions were performed on single-strand DNA templates using the M13K07 helper phage according to published procedures (ref. 17) with the Deaza G/A sequencing mixes (Pharmacia) and $d^{35}ATP$ or $d^{35}CTP$ radio-isotopes. Reactions were analysed on 6% denaturing gels. The inserts of the genomic clones were mapped with several restriction enzymes and displayed similar patterns, except some inserts were longer than others. One of these clones, pGECO 90 (as shown in Figure 6), was selected for further characterization. Figure 7 shows the restriction map of the insert of pGEC0 90 and how it corresponds to the A2 related cDNAs. The restriction enzymes shown in Figure 7 are S: Sal I, P:

Pst I, O: Xho I, X: Xba I, E: Eco RI, M: Sma I. Plasmid pGECO 90 contained unique sites for Sal I and Xba I, but no Cla I site, and this was consistent with the Southern blot analysis shown in Figure 5. The DNA sequence flanking the Eco RI site on this genomic clone was determined and shown to correspond exactly to the related portion of the A8 cDNA, confirming that this fragment represented one unit of the tandem array.

The DNA sequence of the 1.9 kb Xho I/ Eco RI fragment of the pGECO 90 genomic clone corresponding to the 3.5 kb A2 transcript was determined (Figure 8) and compared to the cDNA's sequences. The longest open reading frame (ORF II) found was contained in the Xho I/ Xba I 1.1 kb fragment and potentially encoded a 22 kD protein product (A2 protein). Stop codons were observed in two other frames and upstream from the initiating ATG. Most of this predicted A2 protein was composed of a repetitive sequence consisting of a stretch of ten amino acids repeated nineteen times. Since each unit of this repeat contains two serines, two valines, two leucines and two prolines separated from each other by five residues, the repeated region could also be considered as a stretch of five amino acids repeated thirty-eight times. The only hydrophobic domain was located at the amino terminal portion and may correspond to a signal peptide. The predicted amino acid sequence was compared with proteins reported in the Swiss-Prot database version using a Fasta algorithm (Canada Institute for Scientific and Technical Information: Scientific Numeric Database Service). The most striking identity was observed with an S-antigen of Plasmodium falciparum Vietnamese isolate VI. The alignment of the A2 protein sequence (A2) with the amino-terminal portion of the S-antigen of P. falciparum isolate VI is shown in Figure 9. Identical residues are indicated by dashes and homologous amino acids by dots. As with the L. donovani A2 protein, the

carboxy-terminal portion of this antigen of P. falciparum Vietnamese isolate IV is composed of a stretch of eleven amino acids repeated nineteen times. The repeated units of both proteins are 50% identical and 80% homologous. The S-antigen, as the CS-antigens of Plasmodium, are proteins which are stage-specific, being expressed in the mammalian host but not in the insect host. Therefore, the A2 and S-antigen genes from unrelated human infectious protozoa are expressed specifically in the mammalian host and encode similar proteins. Thus, the A2 and S-antigen proteins may perform similar functions and may be required to enable these protozoa to survive in humans and functional disablement of the A2 sequences in L. donovani may be expected to result in a non-infective promastigote useful as a live attenuated vaccine for leishmaniasis.

Example 5

This Example describes the functional disablement of differentially expressed genes in Leishmania.

One approach for the development of attenuated strains of Leishmania is to functionally disable amastigote-specific genes (such as the A2 gene) from the Leishmania genome (by for example deletion) using homologous recombination. Deletion of genes from protozoa (such as Leishmania) has been described (ref. 18). This procedure involves cloning DNA fragments 5'- and 3'- to the A2 gene and constructing a plasmid vector that contains these flanking DNA sequences sandwiching a neomycin resistance gene. This 5'- neo 3'- fragment of DNA then is used to transform L. donovani promastigotes to G418 resistance. L. donovani is diploid and deletion one allele of the A2 gene in such G418 resistant strains can be determined by Southern blot hybridization using A2 specific probes. The second A2 allele then can be deleted by constructing a second deleting vector containing the 5'- and 3'- A2 flanking sequences

sandwiching a hygromycin resistance gene. Following transformation colonies are selected on medium containing G418 and hygromycin. Deletion of both copies of the A2 gene can be confirmed by Southern blot hybridization.

Example 6

This Example describes the expression of the L. donovani amastigote-specific A2 gene and the recognition of the A2 gene product by kala-azar immune sera.

To produce the A2 protein in a heterologous system, the coding region from the initiating ATG to the Xba I restriction site (see Figure 8) was subcloned in the pET 16B expression vector in frame with the HIS-TAG (Figure 10). The A2 fusion protein of 27 kD was produced in an in vitro transcription-translation assay (TNT system, Promega) using the pET16b/ORF II plasmid and a negative control pBluescript/p53 plasmid, encoding the human p53 protein. The in vitro translated HIS-TAG/A2 ^{35}S-labelled protein was immunoprecipitated with kala-azar immune serum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 11). Kala-azar is a term used to describe the disease caused by L. donovani. The kala-azar immune serum was obtained from a patient suffering from visceral leishmaniasis and reacted strongly against L. donovani antigens on ELISA. In Figure 11, Lanes 1 and 2 contained the labelled proteins A2 and p53, respectively, prior to immunoprecipitation analysis. Lanes 3 and 4 contained proteins A2 and p53, respectively, immunoprecipitated with the kala-azar immune serum (L1) and Lanes 5 and 6 contained proteins A2 and p53, respectively, immunoprecipitated with a control human serum (TXC). The kala-azar serum did not react against the negative control protein human p53 but did immunoprecipitate the A2 gene-product. Neither of the proteins were immunoprecipitated by the control human serum. This analysis showed that the product of the L. donovani A2

gene was specifically recognized by kala-azar immune serum.

To confirm the specificity of the immune reaction, the pET 16b/ORF II plasmid coding for the recombinant A2 fusion protein and a negative control plasmid pET 16b with no insert, were introduced into E. coli. Expression was induced with IPTG, and total lysates of the recombinant E. coli cells separated by SDS-PAGE and analyzed by Western blot analysis using the kala-azar immune serum described above (see Figure 12). In Figure 12, Lane 1 contained E. coli/pET 16b cells and Lane 2 contained E. coli/pET 16b/ORF II cells. The kala-azar serum reacted specifically with protein products of 27.5 and 25 kD in the lysates of cells containing the pET 16b/ORF II plasmid (Lane 2). The 25 kD protein probably corresponded to the A2 protein without the HIS-TAG since the A2 sequence did contain its own initiating ATG. The serum did not react specifically with protein from E. coli lysates containing the control pET 16b plasmid (Lane 1). These data confirmed that the ORF II of the A2 gene encoded a L. donovani protein (A2) that was antigenic in patients with visceral leishmaniasis.

Example 7

This Example describes the Southern blot analysis of the isolated chromosomes of different species and subspecies of Leishmania.

Leishmania strains were obtained from American type Culture Collection, Rockville, Maryland, identified by their accession numbers as follows:

		ATCC
SF-2211:	L. donovani donovani, strain MHOM/IN/80/DD8	50212
SF-1881:	L. donovani infantum	50134
SF-1880:	L. donovani chagasi	50133
SF-1882:	L. Braziliensis Braziliensis	50135
SF-1913:	L. Braziliensis panamensis	50158
SF-1871:	L. Braziliensis Guyanensis	50126

SF-1878: L. Mexicana amazonensis	50131
SF-1911: L. Mexicana mexicana	50156
SF-1864: L. Major	50122
SF-1876: L. tropica	50129
SF-1861: L. aetiopica	50119

Sample blocks were initially prepared from the Leishmania strains, by the following protocol:

1) Promastigote cells were washed once in Hepes-NaCl buffer (21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4 and 6 mM glucose) and resuspended at a density of 5 x 10^8 in the same buffer.

2) Cells were diluted with 1 vol. of 1% low melting point agarose and 100 ul samples were allowed to cool down in sample holders at 4°C.

3) Blocks were transferred into lysis buffer (0.5 M EDTA pH 9.5, 1% sodium lauryl sarcosyl and 2 mg/ml of proteinase K) and incubated at 50°C for 18 h.

4) Blocks were kept at 4°C in 0.5 M EDTA.

Chromosomes were then separated from the sample blocks by Transverse Alternating Field Electrophoresis (TAFE) using a Geneline II System (Beckman instruments) under the following conditions:

- 1% agarose gel were prepared in 1X TAFE buffer (20X TAFE buffer consists in 0.45 M Tris-borate and 0.01 M EDTA). Electrophoresis were carried out at 350 mA for 36 h at 15°C.
- Electrophoresis conditions were:
  - Stage 1: 12 h, 40 s pulse time
  - Stage 2: 12 h, 100 s pulse time
  - Stage 3: 12 h, 160 s pulse time

Southern blots were then prepared from the chromosomal DNA by the following protocol:

1) Gels was soaked in 0.25 M HCl for 15 min. for a partial depurination of DNA.

2) DNA was denatured by an alkaline treatment (gels were soaked 0.5 N NaOH, 1.5 M NaCl for 45 min. with gentle shaking).

3) Gels were neutralized by soaking in 0.5 M Tris-Cl pH 7.0, 3 M NaCl for 45 min.

4) DNA was transferred to nylon membrane using a Vacugene XL (Pharmacia-LKB) for 2 h at 60 mbar in 10X SSC (1X SSC consists in 0.15 M NaCl, 0.015 M sodium citrate).

Hybridization next was carried out, as follows:

- Nylon membranes were prehybridized in 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate for 18 h. at 65°C for 2 h.
- Denatured probes were directly added to the hybridization buffer and blots were incubated for 18 h at 65°C.
- Membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 20 min. and twice in 0.5X SSC, 0.1% SDS for 20 min.
- Membranes were exposed on Kodak X-OMAT films with intensifying screens for 18 h.

The DNA probe used consisted for a pET16b/ORF II 1.1 kb Bam HI fragment agarose gel purified and labelled to high specificity with ^{32}P-dCTP (ICN;3000 ci/mMol) by nick-translation. This fragment contained the complete A2 protein coding region of the L. donovani A2 gene. The Southern blots obtained are shown in Figure 13.

The data provided by Figure 13 shows that the L. donovani A2 gene is present in all three species of L. donovani tested and two subspecies of L. mexicana. However, the A2 coding sequence was not found in L. tropica, L. major, L. braziliensis or L. aetiopica. From these results, it is apparent that the L. donovani A2 gene DNA is useful as a probe to detect specifically L. donovani and L. mexicana among Leishmania species. The L. donovani and L. mexicana species are usually

encountered at widely-different geographical locations, so the probe is specific for infection by the species present in a specific geographical location.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides differentially expressed genes and proteins of Leishmania, including the A2 gene expressed at significantly higher levels in the amastigote stage of the life cycle when the Leishmania organism is present in macrophages than in the promastigote stage. Modifications are possible within the scope of this invention.

REFERENCES

1. WHO, Tropical Disease Report, 1989. pp 85-92.
2. Turco, S.J., and Descoteaux, A. 1992. The Lipophosphoglycan of Leishmania parasites. Annu. Rev. Microbiol. **46**:65-94.
3. Sacks, D.L. 1989. Metacyclogenesis in Leishmania promastigotes. Exp. Parasitology. **69**:100-103.
4. Sacks D.L., and da Silva, R.P. 1987. The generation of infective stage L. major promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. J. Immunol. **139**:3099-3106.
5. Sacks, D.L., Brodin T.N., Turco, S.J. 1990. Developmental modification of the lipophosphoglycan from L. Major promastigotes during metacyclogenesis. Mol. Biochemical Parasitol. **42**:225-234.
6. Medina-Acosta, E., Karess, R.E., Schwartz H., and Russell, D.G. 1989. The promastigote surface protease (gp63) of Leishmania is expressed but differentially processed and localized in the amastigote stage. Mol. Biochemical Parasitol. **37**:263-274.
7. Turco, S.J. and Sacks, D.L. 1991. Expression of stage-specific lipophosphoglycan in Leishmania major amastigotes. Mol. Biochemical Parasitol. **45**:91-100.
8. McConville, M.J., and Blackwell J.M. 1991. Developmental changes in the glycosylated phosphatidylinositols of L. donovani J. Biol. Chem. **260**:15170-15179.
9. Bogdan, C., Röllinghoff M., and Solbach, W. 1990. Evasion strategies of Leishmania parasites. Parasitol. Today. **6**:183-187.

10. Modabber, F. 1989. Experiences with vaccines against cutaneous leishmaniasis: of men and mice. Parasitol. 98:S49-S60.

11. Joshi, M., Dwyer, D.M., and Nakhasi, H.L. 1993. Cloning and characterization of differentially expressed genes from in vitro-grown "amastigotes" of Leishmania donovani. Mol. Biochemical Parasitol. 58:345-354.

12. Descoteaux, A., and Matlashewski, G. 1989. c-fos and tumor necrosis factor gene expression in Leishmania donovani-infected macrophages. Mol.Cell. Biol. 9:5223-5227.

13. Doyle, P.S. Engel, J.C., Pimenta, P.F.P. da Silva, P. and Dwyer. 1991. Leishmania donovani: Long-term culture of axenic amastigotes at 37°C. Exp. Parasitol. 73:326-334.

14. Sambrook, J., Fritsch, E.F., and Maniatis. 1989. Molecular cloning. A laboratory guide. Cold Spring Harbor Laboratories Press, New York. pp 7.26-7.29.

15. Sambrook, J., Fritsch, E.F., and Maniatis. 1989. Molecular cloning. A laboratory guide. Cold Spring Harbor Laboratories Press, New York. pp 10.44-10.45.

16. Sambrook, J., Fritsch, E.F., and Maniatis. 1989. Molecular cloning. A laboratory guide. Cold Spring Harbor Laboratories Press, New York. pp 9.38-9.40.

17. Sambrook, J., Fritsch, E.F., and Maniatis. 1989. Molecular cloning. A laboratory guide. Cold Spring Harbor Laboratories Press, New York. pp 4.48.

18. Cruz,A., and Beverley, S.M. 1990. Gene-replacement in parasitic protozoa. Nature 348:171-173.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(B) STREET: 845 Sherbrooke Street West
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(D) STATE: Quebec
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): H3V 4P6

(ii) TITLE OF INVENTION: DIFFERENTIALLY EXPRESSED LEISHMANIA GENES AND PROTEINS

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1091 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGCTCCCCC AGCGACCCTC TCGGCAACGC GAGCGCCCCA GTCCCCCCAC GCACAACTTT 60

GACCGAGCAC AATGAAGATC CGCAGCGTGC GTCCGCTTGT GGTGTTGCTG GTGTGCGTCG 120

CGGCGGTGCT CGCACTCAGC GCCTCCGCTG AGCCGCACAA GGCGGCCGTT GACGTCGGCC 180

CGCTCTCCGT TGGCCCGCAG TCCGTCGGCC CGCTCTCTGT TGGCCCGCAG GCTGTTGGCC 240

CGCTCTCCGT TGGCCCGCAG TCCGTCGGCC CGCTCTCTGT TGGCCCGCAG GCTGTTGGCC 300

CGCTCTCTGT TGGCCCGCAG TCCGTTGGCC CGCTCTCCGT TGGCCCGCTC TCCGTTGGCC 360

CGCAGTCTGT TGGCCCGCTC TCCGTTGGCT CGCAGTCCGT CGGCCCGCTC TCTGTTGGTC 420

CGCAGTCCGT CGGCCCGCTC TCCGTTGGCC CGCAGGCTGT TGGCCCGCTC TCCGTTGGCC 480

CGCAGTCCGT CGGCCCGCTC TCTGTTGGCC CGCAGGCTGT TGGCCCGCTC TCTGTTGGCC 540

CGCAGTCCGT TGGCCCGCTC TCCGTTGGCC CGCAGTCTGT TGGCCCGCTC TCCGTTGGCT 600

CGCAGTCCGT CGGCCCGCTC TCTGTTGGTC CGCAGTCCGT CGGCCCGCTC TCCGTTGGCC 660

CGCAGTCTGT CGGCCCGCTC TCCGTTGGCC CGCAGTCCGT CGGCCCGCTC TCCGTTGGTC 720

CGCAGTCCGT TGGCCCGCTC TCCGTTGGCC CGCAGTCCGT TGACGTTTCT CCGGTGTCTT 780

AAGGCTCGGC GTCCGCTTTC CGGTGTGCGT AAAGTATATG CCATGAGGCA TGGTGACGAG 840

GCAAACCTTG TCAGCAATGT GGCATTATCG TACCCGTGCA AGAGCAACAG CAGAGCTGAG 900

TGTTCAGGTG GCCACAGCAC CACGCTCCTG TGACACTCCG TGGGGTGTGT GTGACCTTGG 960

CTGCTGTTGC CAGGCGGATG AACTGCGAGG GCCACAGCAG CGCAAGTGCC GCTTCCAACC 1020

TTGCGACTTT CACGCCACAG ACGCATAGCA GCGCCCTGCC TGTCGCGGCG CATGCGGGCA 1080

AGCCATCTAG A 1091

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGAAGATCC GCAGCGTGCG TCCGCTTGTG GTGTTGCTGG TGTGCGTCGC GGCGGTGCTC 60

GCACTCAGCG CCTCCGCTGA GCCGCACAAG GCGGCCGTTG ACGTCGGCCC GCTCTCCGTT 120

GGCCCGCAGT CCGTCGGCCC GCTCTCTGTT GGCCCGCAGG CTGTTGGCCC GCTCTCCGTT 180

GGCCCGCAGT CCGTCGGCCC GCTCTCTGTT GGCCCGCAGG CTGTTGGCCC GCTCTCTGTT 240

GGCCCGCAGT CCGTTGGCCC GCTCTCCGTT GGCCCGCTCT CCGTTGGCCC GCAGTCTGTT 300

GGCCCGCTCT CCGTTGGCTC GCAGTCCGTC GGCCCGCTCT CTGTTGGTCC GCAGTCCGTC 360

GGCCCGCTCT CCGTTGGCCC GCAGGCTGTT GGCCCGCTCT CCGTTGGCCC GCAGTCCGTC 420

GGCCCGCTCT CTGTTGGCCC GCAGGCTGTT GGCCCGCTCT CTGTTGGCCC GCAGTCCGTT 480

GGCCCGCTCT CCGTTGGCCC GCAGTCTGTT GGCCCGCTCT CCGTTGGCTC GCAGTCCGTC 540

GGCCCGCTCT CTGTTGGTCC GCAGTCCGTC GGCCCGCTCT CCGTTGGCCC GCAGTCTGTC 600

GGCCCGCTCT CCGTTGGCCC GCAGTCCGTC GGCCCGCTCT CCGTTGGTCC GCAGTCCGTT 660

GGCCCGCTCT CCGTTGGCCC GCAGTCCGTT GACGTTTCTC CGGTGTCTTA A 711

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 236 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Lys Ile Arg Ser Val Arg Pro Leu Val Val Leu Leu Val Cys Val
1 5 10 15

Ala Ala Val Leu Ala Leu Ser Ala Ser Ala Glu Pro His Lys Ala Ala
20 25 30

Val Asp Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu
35 40 45

Ser Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser
50 55 60

Val Gly Pro Leu Ser Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val
65 70 75 80

Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Leu Ser Val Gly
85 90 95

Pro Gln Ser Val Gly Pro Leu Ser Val Gly Ser Gln Ser Val Gly Pro
100 105 110

Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln
115 120 125

Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser
130 135 140

Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val
145 150 155 160

Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly
165 170 175

Ser Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro
180 185 190

Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln
195 200 205

Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser
210 215 220

Val Gly Pro Gln Ser Val Asp Val Ser Pro Val Ser
225 230 235

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 269 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly
1               5                  10                  15

Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly
            20                  25                  30

Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu
        35                  40                  45

Gly Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr
    50                  55                  60

Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly Gly Pro Gly Ser
65                  70                  75                  80

Glu Gly Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly
                85                  90                  95

Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly Gly Pro Gly
            100                 105                 110

Ser Glu Gly Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys
        115                 120                 125

Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly Gly Pro
    130                 135                 140

Gly Ser Glu Gly Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro
145                 150                 155                 160

Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly Gly
                165                 170                 175

Pro Gly Ser Glu Ser Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly
            180                 185                 190

Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Gly Pro Lys Gly Thr Gly
        195                 200                 205

Pro Lys Gly Thr Gly Gly Pro Gly Ser Glu Ala Gly Thr Glu Gly Pro
    210                 215                 220

Lys Gly Thr Gly Gly Pro Gly Ser Glu Ala Gly Thr Glu Gly Pro Lys
225                 230                 235                 240

Gly Thr Gly Gly Pro Gly Ser Gly Gly Glu His Ser His Asn Lys Lys
                245                 250                 255

Lys Ser Lys Lys Ser Ile Met Asn Met Leu Ile Gly Val
            260                 265
```

CLAIMS

What we claim is:

1. An isolated and purified chromosomal DNA molecule, the molecule comprising at least a portion coding for a differentially expressed gene of a Leishmania organism, the differentially expressed gene being expressed at an increased level when the amastigote form of the Leishmania organism is present within a macrophage in comparison to the promastigote form of the Leishmania organism.

2. The DNA molecule of claim 1 wherein the increased level is at least about a ten fold increase.

3. The DNA molecule of claim 1 wherein the differentially expressed gene is a virulence gene of the Leishmania organism.

4. The DNA molecule of claim 3 wherein the virulence gene is required for maintenance of infection by the amastigote form of the Leishmania organism.

5. The DNA molecule of claim 1 wherein the differentially expressed gene encodes a protein.

6. The DNA molecule of claim 1 wherein the Leishmania organism is Leishmania donovani.

7. The DNA molecule of claim 1 wherein the differentially expressed gene has the DNA coding sequence set out in Figure 8 or its complementary strand or a DNA molecule coding for a differentially expressed gene of a Leishmania organism which hybridizes under stringent conditions thereto.

8. An isolated and purified DNA fragment having the nucleotide sequence:

GAGCTCCCCC AGCGACCCTC TCGGCAACGC GAGCGCCCCA GTCCCCCCAC GCACAACTTT 60
GACCGAGCAC AATGAAGATC CGCAGCGTGC GTCCGCTTGT GGTGTTGCTG GTGTGCGTCG 120
CGGCGGTGCT CGCACTCAGC GCCTCCGCTG AGCCGCACAA GGCGGCCGTT GACGTCGGCC 180
CGCTCTCCGT TGGCCCGCAG TCCGTCGGCC CGCTCTCTGT TGGCCCGCAG GCTGTTGGCC 240
CGCTCTCCGT TGGCCCGCAG TCCGTCGGCC CGCTCTCTGT TGGCCCGCAG GCTGTTGGCC 300
CGCTCTCTGT TGGCCCGCAG TCCGTTGGCC CGCTCTCCGT TGGCCCGCTC TCCGTTGGCC 360
CGCAGTCTGT TGGCCCGCTC TCCGTTGGCT CGCAGTCCGT CGGCCCGCTC TCTGTTGGTC 420
CGCAGTCCGT CGGCCCGCTC TCCGTTGGCC CGCAGGCTGT TGGCCCGCTC TCCGTTGGCC 480
CGCAGTCCGT CGGCCCGCTC TCTGTTGGCC CGCAGGCTGT TGGCCCGCTC TCTGTTGGCC 540

```
CGCAGTCCGT TGGCCCGCTC TCCGTTGGCC CGCAGTCTGT TGGCCCGCTC TCCGTTGGCT  600
CGCAGTCCGT CGGCCCGCTC TCTGTTGGTC CGCAGTCCGT CGGCCCGCTC TCCGTTGGCC  660
CGCAGTCTGT CGGCCCGCTC TCCGTTGGCC CGCAGTCCGT CGGCCCGCTC TCCGTTGGTC  720
CGCAGTCCGT TGGCCCGCTC TCCGTTGGCC CGCAGTCCGT TGACGTTTCT CCGGTGTCTT  780
AAGGCTCGGC GTCCGCTTTC CGGTGTGCGT AAAGTATATG CCATGAGGCA TGGTGACGAG  840
GCAAACCTTG TCAGCAATGT GGCATTATCG TACCCGTGCA AGAGCAACAG CAGAGCTGAG  900
TGTTCAGGTG GCCACAGCAC CACGCTCCTG TGACACTCCG TGGGGTGTGT GTGACCTTGG  960
CTGCTGTTGC CAGGCGGATG AACTGCGAGG GCCACAGCAG CGCAAGTGCC GCTTCCAACC 1020
TTGCGACTTT CACGCCACAG ACGCATAGCA GCGCCCTGCC TGTCGCGGCG CATGCGGGCA 1080
AGCCATCTAG A                                                      1091
```

(SEQ ID NO: 1), or its complementary strand, or a DNA moleucle coding for a differentially-expressed gene of a Leishmania organism which hybridizes thereto under stringent conditions.

9. An isolated and purified DNA fragment having the nucleotide sequence:

```
ATGAAGATCC GCAGCGTGCG TCCGCTTGTG GTGTTGCTGG TGTGCGTCGC GGCGGTGCTC   60
GCACTCAGCG CCTCCGCTGA GCCGCACAAG GCGGCCGTTG ACGTCGGCCC GCTCTCCGTT  120
GGCCCGCAGT CCGTCGGCCC GCTCTCTGTT GGCCCGCAGG CTGTTGGCCC GCTCTCCGTT  180
GGCCCGCAGT CCGTCGGCCC GCTCTCTGTT GGCCCGCAGG CTGTTGGCCC GCTCTCTGTT  240
GGCCCGCAGT CCGTTGGCCC GCTCTCCGTT GGCCCGCTCT CCGTTGGCCC GCAGTCTGTT  300
GGCCCGCTCT CCGTTGGCTC GCAGTCCGTC GGCCCGCTCT CTGTTGGTCC GCAGTCCGTC  360
GGCCCGCTCT CCGTTGGCCC GCAGGCTGTT GGCCCGCTCT CCGTTGGCCC GCAGTCCGTC  420
GGCCCGCTCT CTGTTGGCCC GCAGGCTGTT GGCCCGCTCT CTGTTGGCCC GCAGTCCGTT  480
GGCCCGCTCT CCGTTGGCCC GCAGTCTGTT GGCCCGCTCT CCGTTGGCTC GCAGTCCGTC  540
GGCCCGCTCT CTGTTGGTCC GCAGTCCGTC GGCCCGCTCT CCGTTGGCCC GCAGTCTGTC  600
GGCCCGCTCT CCGTTGGCCC GCAGTCCGTC GGCCCGCTCT CCGTTGGTCC GCAGTCCGTT  660
GGCCCGCTCT CCGTTGGCCC GCAGTCCGTT GACGTTTCTC CGGTGTCTTA A           711
```

(SEQ ID NO: 2), or its complementary strand, or a DNA molecule coding for a differentially-expressed gene of a Leishmania organism which hybridizes thereto under stringent conditions.

10. An isolated and purified DNA fragment encoding the amino acids sequence:

```
Met Lys Ile Arg Ser Val Arg Pro Leu Val Val Leu Leu Val Cys Val
1               5                  10                  15
Ala Ala Val Leu Ala Leu Ser Ala Ser Ala Glu Pro His Lys Ala Ala
            20                  25                  30
Val Asp Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu
        35                  40                  45
```

```
Ser Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser
    50                  55                  60
Val Gly Pro Leu Ser Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val
65                  70                  75                  80
Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Leu Ser Val Gly
                85                  90                  95
Pro Gln Ser Val Gly Pro Leu Ser Val Gly Ser Gln Ser Val Gly Pro
            100                 105                 110
Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln
        115                 120                 125
Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser
    130                 135                 140
Val Gly Pro Gln Ala Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val
145                 150                 155                 160
Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly
                165                 170                 175
Ser Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro
            180                 185                 190
Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser Val Gly Pro Gln
        195                 200                 205
Ser Val Gly Pro Leu Ser Val Gly Pro Gln Ser Val Gly Pro Leu Ser
    210                 215                 220
Val Gly Pro Gln Ser Val Asp Val Ser Pro Val Ser
225                 230                 235
```

(SEQ ID NO: 3), or its complementary strand, or a DNA molecule coding for a differentially-expressed gene of a Leishmania organism which hybridizes thereto under stringent conditions.

11. A recombinant plasmid adapted for transformation of a microbial host, the recombinant plasmid comprising a plasmid vector into which a DNA segment comprising the purified and isolated DNA molecule of any one of claims 1 to 10 has been inserted.

12. The recombinant plasmid of claim 11 which is plasmid pGECO 90 having ATCC accession number 75510.

13. A purified protein encoded by a differentially expressed gene of a Leishmania organism, the differentially expressed gene being expressed at an increased level when the amastigote form of the Leishmania organism is present within a macrophage.

14. The purified protein of claim 13 wherein the increased level is at least about a ten fold increase.

15. The protein of claim 13 wherein the differentially expressed gene is a virulence gene of the Leishmania organism.

16. The protein of claim 15 wherein the virulence gene is required for maintenance of infection by the amastigote form of the Leishmania organism.

17. The protein of claim 13 wherein the differentially expressed gene has the DNA sequence set out in Figure 8 or its complementary strand, or a DNA sequence coding for a differentially expressed gene of a Leishmania organism which hybridizes under stringent conditions thereto.

18. The protein of claim 13 wherein the Leishmania organism is Leishmania donovani.

19. An attenuated strain of Leishmania wherein the virulence gene has been functionally disabled.

20. The attenuated strain of claim 19 wherein the virulence gene has been functionally disabled by deletion.

21. The attenuated strain of claim 19 wherein the virulence gene has been functionally disabled by mutagenesis thereof.

22. The attenuated strain of claim 21 wherein the virulence gene has been functionally disabled by insertional mutagenesis.

23. The attenuated strain of claim 19 wherein the differentially expressed virulence gene has the DNA sequence set out in Figure 8 or its complementary strand, or a DNA sequence coding for a differentially expressed gene of a Leishmania organism which hybridizes under stringent conditions thereto.

24. A vaccine to provide protective immunity to a host against disease caused by a Leishmania organism, comprising an effective amount of the protein claimed in claim 13 and a physiologically-acceptable carrier therefor.

25. The vaccine of claim 24 wherein the carrier comprises an adjuvant.

26. The vaccine of claim 24 wherein the protein is presented to the immune system of the host in combination with an ISCOM or a liposome.

27. A live vaccine to provide protective immunity to a host against disease caused by a Leishmania organism, comprising an effective amount of the attenuated strain of Leishmania wherein the virulence gene has been functionally disabled and a physiologically-acceptable carrier therefor.

28. The vaccine of claim 24 or 27 formulated to be administered in an injectable form, intranasally or orally.

29. A method of immunizing a host against disease caused by a Leishmania organism, which comprises administering to the host an effective amount of vaccine claimed in any one of claims 24 or 27.

30. An antibody raised against the protein of claim 13.

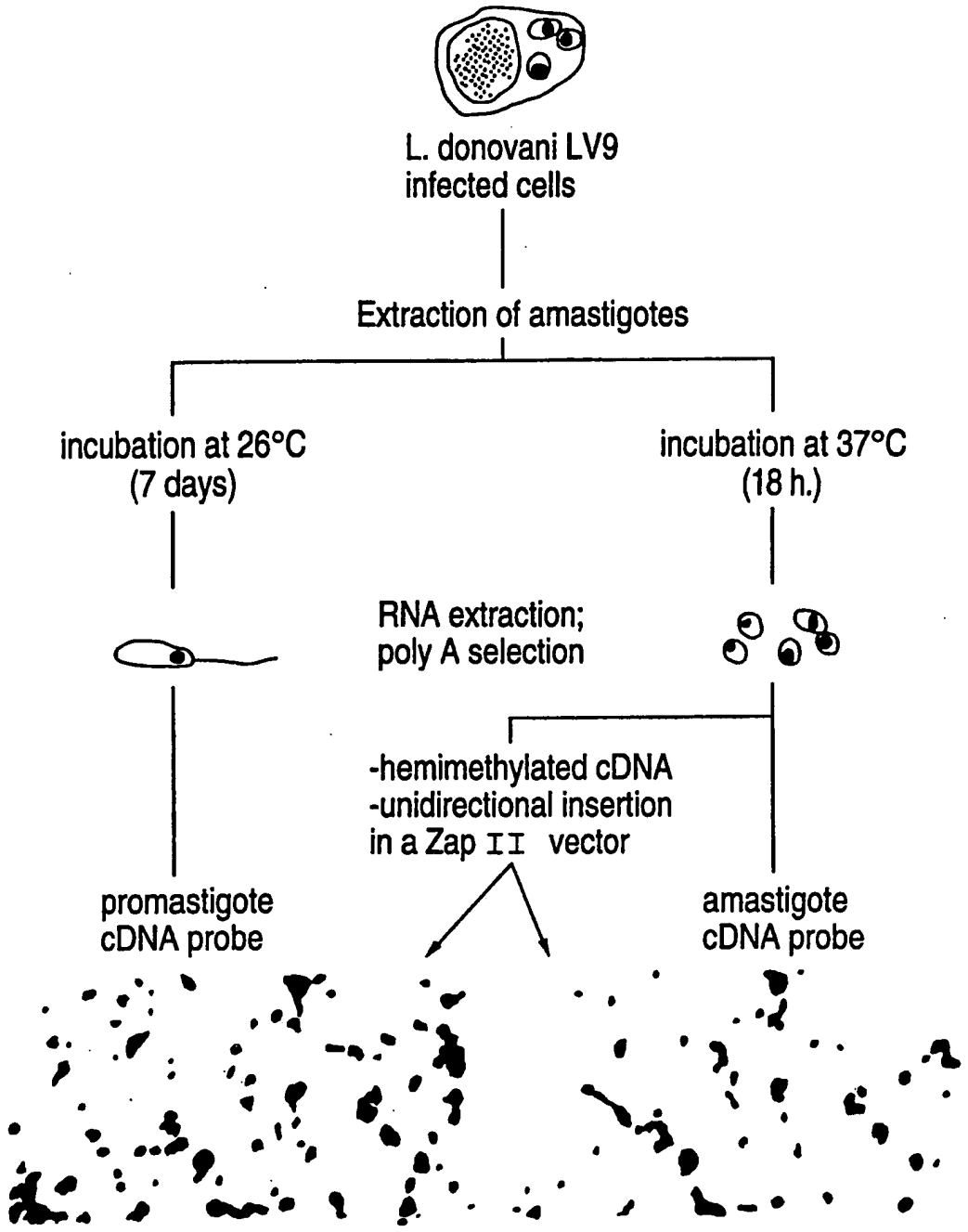

L. donovani LV9
infected cells
Extraction of amastigotes
incubation at 26°C
(7 days)
incubation at 37°C
(18 h.)
RNA extraction;
poly A selection
-hemimethylated cDNA
-unidirectional insertion
in a Zap II vector
promastigote
cDNA probe
amastigote
cDNA probe

FIG.1.

2 3 5 6 8 9 11

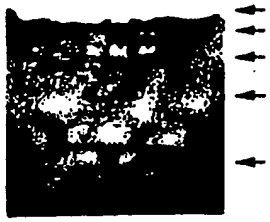


FIG.2.

(probes)

A2

2 3 5 6 8 9 11

A6

2 3 5 6 8 9 11

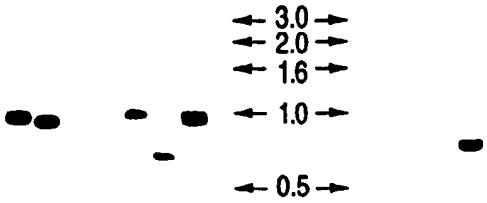


FIG.3.

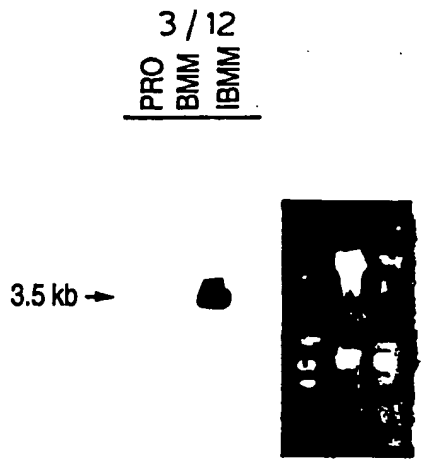


FIG.4.

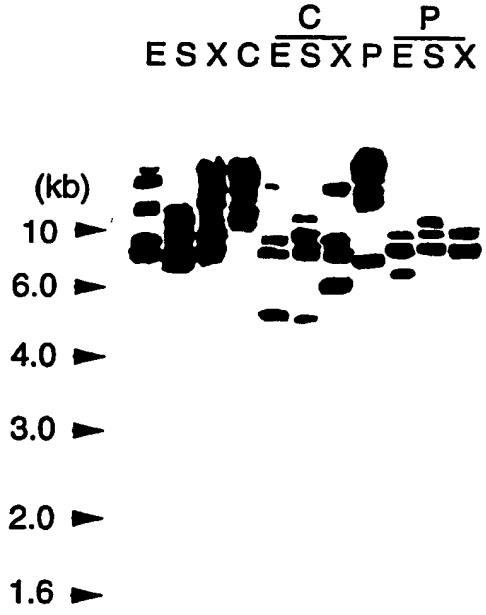


FIG.5.

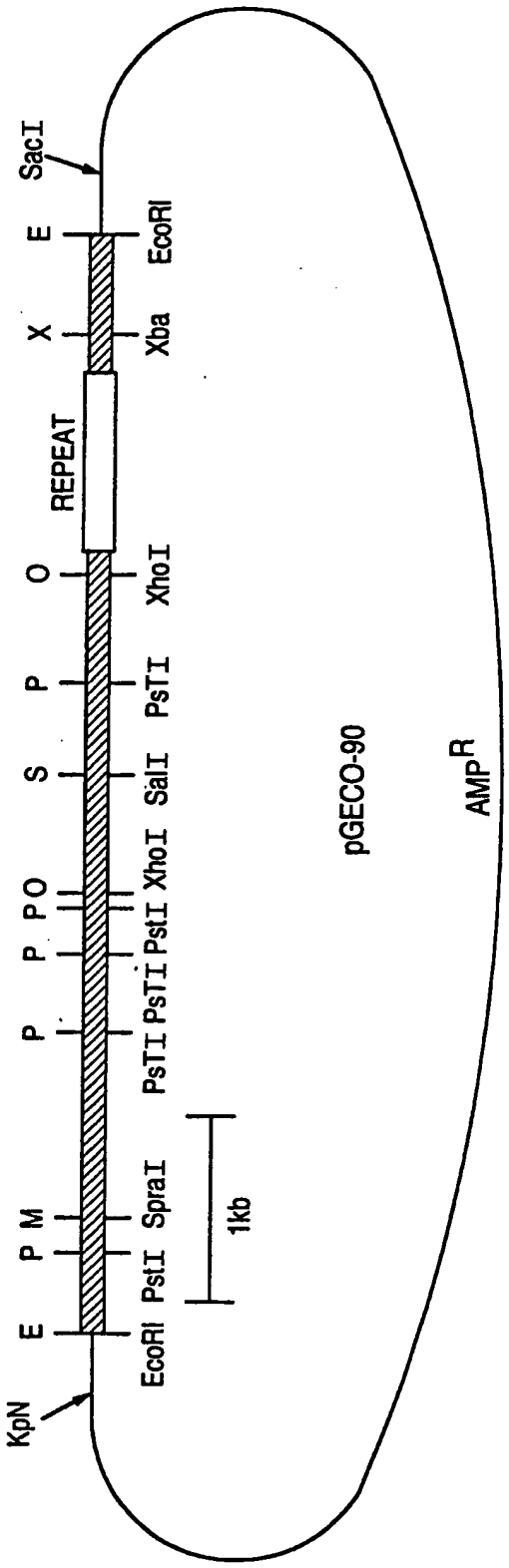

SacI
E
EcoRI
X
Xba
REPEAT
O
XhoI
P
PstI
S
SalI
XhoI
PstI
PstI
PstI
P P P O
SpraI
P M
PstI
E
EcoRI
KpN
1kb
pGECO-90
AMP^R

FIG.6.

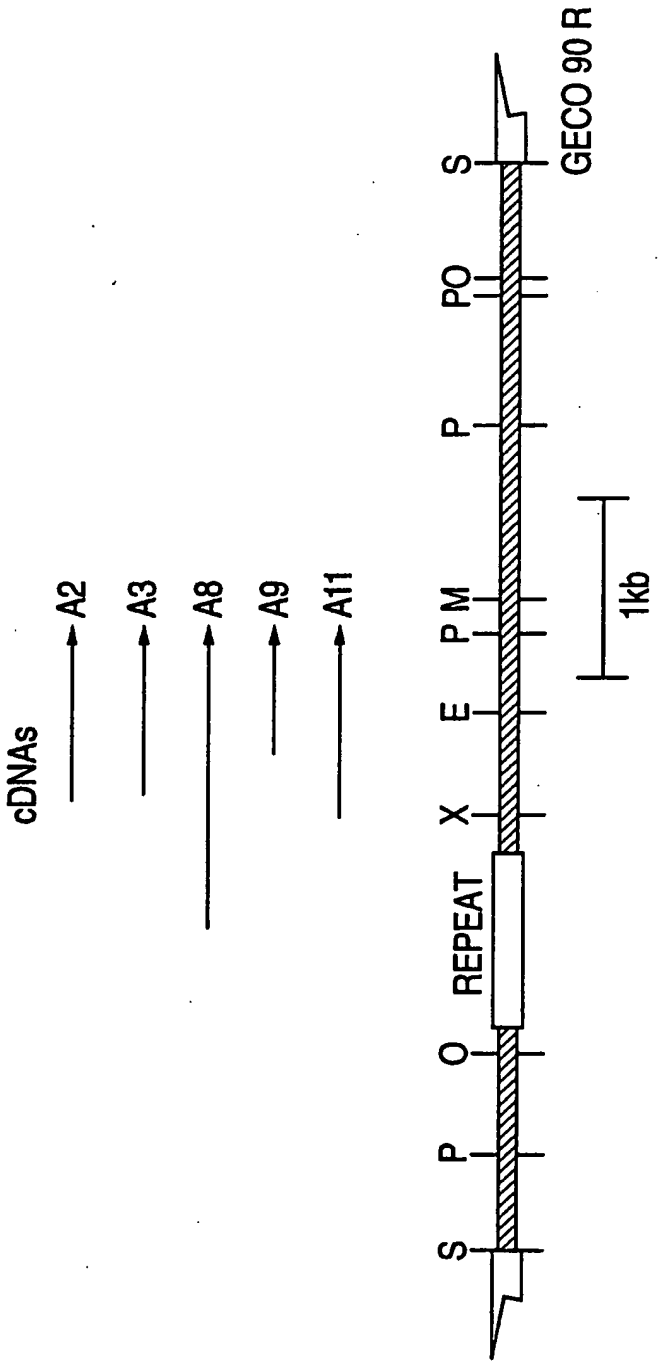


FIG.7

FIG.8A.

XHO I

GAGCTCCCCCAGCGACCCTCTCGGCAACGCGAGCGCCCCAGTCCCCCCACGCACAACTTTGACCGAGCACA

ORF II

Met Lys Ile Arg Ser Val Arg Pro Leu Val Val Leu Leu Val Cys Val Ala Ala Val Leu Ala Leu
1, ATG AAG ATC CGC AGC GTG CGT CCG CTT GTG GTG TTG CTG GTG TGC GTC GCG GCG GTG CTC GCA CTC

Ser Ala Ser Ala Glu Pro His Lys Ala Ala Val Asp
67 AGC GCC TCC GCT GAG CCG CAC AAG GCG GCC GTT GAC

Val Gly Pro Leu Ser Val Gly Pro
103 GTC GGC CCG CTC TCC GTT GGC CCG

Gln Ser Val Gly Pro Leu Ser Val Gly Pro
127 CAG TCC GTC GGC CCG CTC TCT GTT GGC CCG

Gln Ala Val Gly Pro Leu Ser Val Gly Pro
157 CAG GCT GTT GGC CCG CTC TCC GTT GGC CCG

Gln Ser Val Gly Pro Leu Ser Val Gly Pro
187 CAG TCC GTC GGC CCG CTC TCT GTT GGC CCG

Gln Ala Val Gly Pro Leu Ser Val Gly Pro
217 CAG GCT GTT GGC CCG CTC TCC GTT GGC CCG

Gln Ser Val Gly Pro Leu Ser Val Gly Pro Leu Ser Val Gly Pro
247 CAG GCT GTT GGC CCG CTC TCC GTT GGC CCG CTC TCC GTT GGC CCG

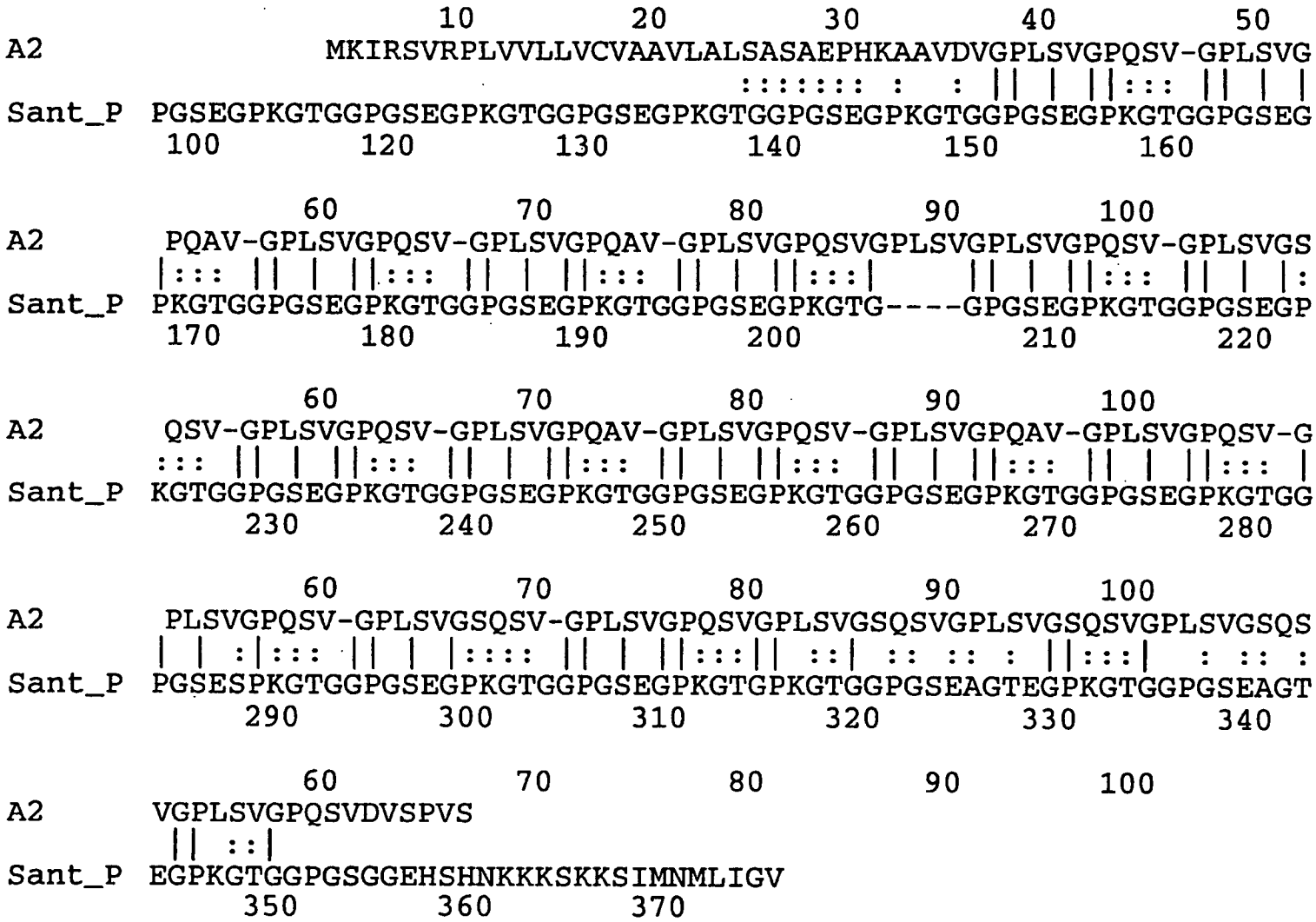
```
      Gln Ser Val Gly Pro Leu Ser Val Gly Ser
  292 CAG TCT GTC GGC CCG CTC TCC GTT GGC TCG

      Gln Ser Val Gly Pro Leu Ser Val Gly Pro
  322 CAG TCC GTC GGC CCG CTC TCT GTT GGT CCG

      Gln Ser Val Gly Pro Leu Ser Val Gly Pro
  352 CAG TCC GTC GGC CCG CTC TCT GTT GGC CCG

      Gln Ala Val Gly Pro Leu Ser Val Gly Pro
  382 CAG GCT GTT GGC CCG CTC TCC GTT GGC CCG

      Gln Ser Val Gly Pro Leu Ser Val Gly Pro
  412 CAG TCC GTC GGC CCG CTC TCT GTT GGC CCG

      Gln Ala Val Gly Pro Leu Ser Val Gly Pro
  442 CAG GCT GTT GGC CCG CTC TCT GTT GGC CCG

      Gln Ser Val Gly Pro Leu Ser Val Gly Pro
  472 CAG TCC GTT GGC CCG CTC TCC GTT GGC CCG

      Gln Ser Val Gly Pro Leu Ser Val Gly Ser
  502 CAG TCT GTT GGC CCG CTC TCC GTT GGC TCG

      Gln Ser Val Gly Pro Leu Ser Val Gly Pro
  532 CAG TCC GTC GGC CCG CTC TCT GTT GGT CCG

      Gln Ser Val Gly Pro Leu Ser Val Gly Pro
  562 CAG TCC GTC GGC CCG CTC TCC GTT GGC CCG
```

FIG.8B.

FIG.8C.

```
                Gln Ser Val Gly Pro Leu Ser Val Gly Pro
            592 CAG TCT GTC GGC CCG CTC TCC GTT GGC CCG

                Gln Ser Val Gly Pro Leu Ser Val Gly Pro
            622 CAG TCC GTC GGC CCG CTC TCC GTT GGT CCG

                Gln Ser Val Gly Pro Leu Ser Val Gly Pro
            652 CAG TCC GTT GGC CCG CTC TCC GTT GGC CCG

                Gln Ser Val
            682 CAG TCC GTC

    Asp Val Ser Pro Val Ser ***
691 GAC GTT TCT CCG GTG TCT TAAGGCTCGGCGTCCGCTTTCCGGTGTGCGTAAAGTATATGCCATGAGGCATGGTGACGAGGCAAAC
776 CTTGTCAGCAATGTGGCATTATCGTACCCGTGCAAGAGCAACAGCAGAGCTGAGTGTTCAGGTGGCCACAGCACCACGCTCCTGTGACACT
867 CCGTGGGGTGTGTGTGACCTTGGCTGCTGTTGCCAGGCGGATGAACTGCGAGGGCCACAGCAGCGCAAGTGCCGCTTCCAACCTTGCGACT
958 TTCACGCCACAGACGCATAGCAGCGCCCTGCCTGTCGCGGCGCATGCGGGCAAGCCATCTAGA
                                                                XBA I
```

FIG.9.


10 20 30 40 50
A2 MKIRSVRPLVVLLVCVAAVLALSASAEPHKAAVDVGPLSVGPQSV-GPLSVG
Sant_P PGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEG
100 120 130 140 150 160
60 70 80 90 100
A2 PQAV-GPLSVGPQSV-GPLSVGPQAV-GPLSVGPQSVGPLSVGPLSVGPQSV-GPLSVGS
Sant_P PKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTG----GPGSEGPKGTGGPGSEGP
170 180 190 200 210 220
60 70 80 90 100
A2 QSV-GPLSVGPQSV-GPLSVGPQAV-GPLSVGPQSV-GPLSVGPQAV-GPLSVGPQSV-G
Sant_P KGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGG
230 240 250 260 270 280
60 70 80 90 100
A2 PLSVGPQSV-GPLSVGSQSV-GPLSVGPQSVGPLSVGSQSVGPLSVGSQSVGPLSVGSQS
Sant_P PGSESPKGTGGPGSEGPKGTGGPGSEGPKGTGPKGTGGPGSEAGTEGPKGTGGPGSEAGT
290 300 310 320 330 340
60 70 80 90 100
A2 VGPLSVGPQSVDVSPVS
Sant_P EGPKGTGGPGSGGEHSHNKKKSKKSIMNMLIGV
350 360 370

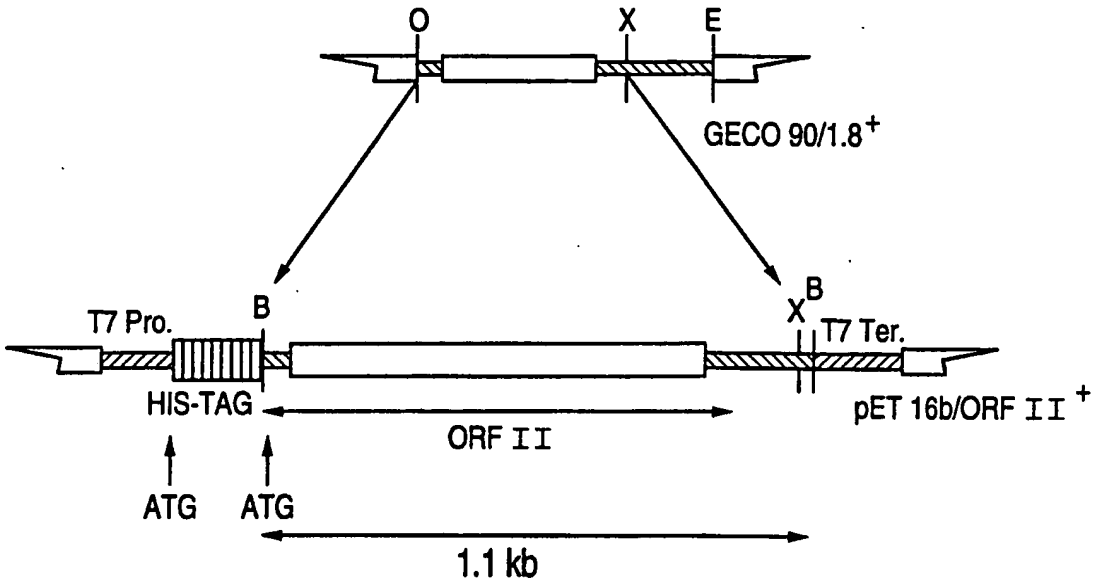

O
X
E
GECO 90/1.8+
B
X
B
T7 Pro.
T7 Ter.
HIS-TAG
ORF II
pET 16b/ORF II +
ATG
ATG
1.1 kb

FIG.10.

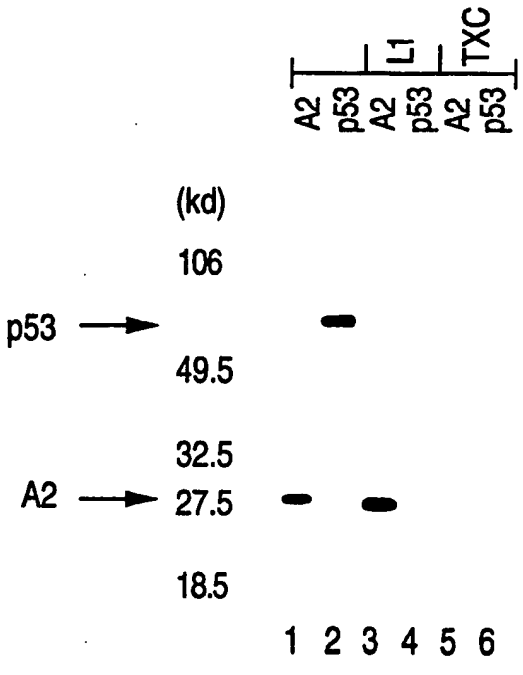

TXC
L1
A2
p53
A2
p53
A2
p53
(kd)
106
p53
49.5
32.5
A2
27.5
18.5
1 2 3 4 5 6

FIG.11.

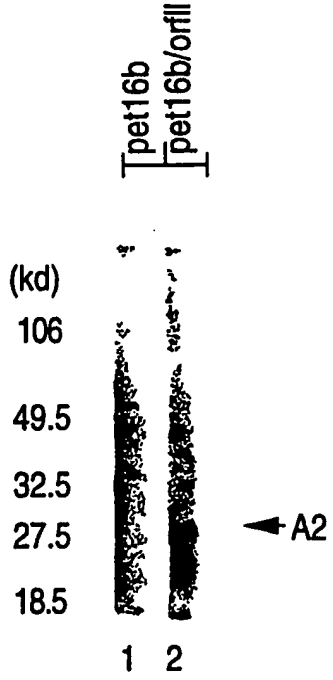


FIG.12.

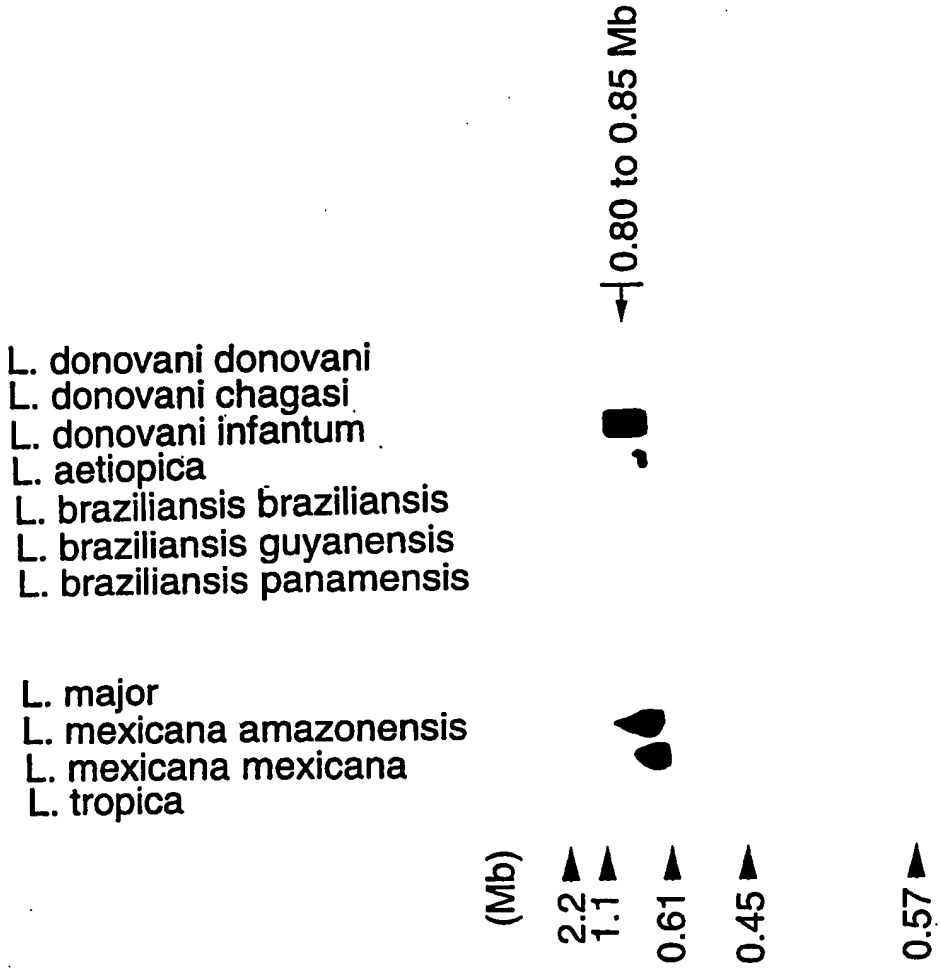

0.80 to 0.85 Mb
L. donovani donovani
L. donovani chagasi
L. donovani infantum
L. aetiopica
L. braziliansis braziliansis
L. braziliansis guyanensis
L. braziliansis panamensis
L. major
L. mexicana amazonensis
L. mexicana mexicana
L. tropica
(Mb)
2.2
1.1
0.61
0.45
0.57

FIG.13.

INTERNATIONAL SEARCH REPORT

Int[redacted] Application No
P[redacted]CA 94/00482

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/30 C12N15/63 C07K14/44 C12N1/10 A61K35/68
A61K38/17 C12P21/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol.58, no.2, 1993 pages 345 - 354 MANJU JOSHI ET AL. 'Cloning and characterization of differentially expressed genes from in vitro-grown 'amastigotes' of Leishmania donovani' cited in the application see abstract see page 348, left column, last paragraph - right column, paragraph 1 see page 349, left column, paragraph 2 - right column, paragraph 1 see page 351, left column, paragraph 2 - right column, paragraph 1 --- -/--	1,5,6, 13,15,18

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 1994	14-12-1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF PROTOZOOLOGY, vol.26, no.3, August 1979 page 49A ESTHER MARVA ET AL. 'Vaccination of mice with chemically attenuated Leishmani tropica' see abstract n. 140 ---	19
A	JOURNAL OF IMMUNOLOGY., vol.140, no.7, 1 April 1988, BALTIMORE US pages 2406 - 2414 ALFRED A. PAN ET AL. 'Monoclonal antibodies specific for the amastigote stage of Leishmania pifanoi. I.Characterization of antigens associated with stage- and species-specific determinants' see abstract see page 2407, left column, paragraph 2 see page 2407, right column, last paragraph - page 2408, right column, paragraph 1 see page 2408, right column, paragraph 4 - page 2409, left column, paragraph 1 see page 2409, right column, paragraph 2 - page 2410, right column, paragraph 1 see page 2411, left column, paragraph 3 - right column, paragraph 1 see page 2412, left column, paragraph 2 - right column, paragraph 1 ---	13,18, 24,29,30
A	INFECTION AND IMMUNITY, vol.36, no.1, April 1982, WASHINGTON US pages 430 - 431 K.P. CHANG ET AL. 'Antigenic changes during intracellular differentiation of Leishmania mexicana in cultured macrophages' see abstract see page 431, left column, paragraph 1 - right column, paragraph 1 --- -/--	13

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MOLECULAR AND CELLULAR BIOLOGY, vol.14, no.5, May 1994 pages 2975 - 2984 HUGUES CHAREST ET AL. 'Developmental gene expression in Leishmania donovani: Differential cloning and analysis of an amastigote-stage-specific gene' see abstract see page 2975, right column, paragraph 3 - page 2976, left column, paragraph 1 see page 2977, right column, paragraph 3 - page 2978, left column, paragraph 1; figure 5 see page 2981, right column, paragraph 2 - page 2982, left column, paragraph 1	1-18

INTERNATIONAL SEARCH REPORT

[redacted]tional application No.
PCT/CA94/00482

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claim 29 is directed to a mehtod of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.